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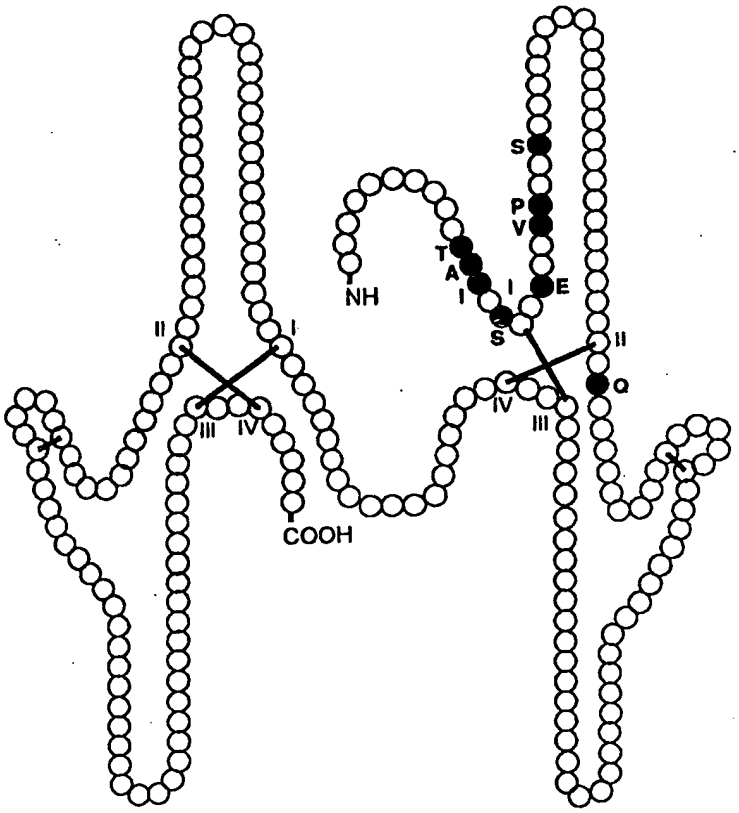
(57) Abstract

The invention refers to an immunogenic region comprising a part of the non-collagenous domain of the α3 chain of type IV collagen having intact cysteine residues, said part being recognized by circulating autoantibodies from Goodpasture patients. This part comprises at least five amino acid residues which are substantially non-adjacent in the amino acid sequence, it is located in the amino terminal end of said α3 chain, and it is exposed to said autoantibodies. The invention also refers to methods for the determination in body fluids of circulating autoantibodies from Goodpasture patients.



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Immunogenic region recognized by circulating autoantibodies from Goodpasture patients

The invention refers to the immunogenic region of the non-collagenous domain of the α3 chain of type IV collagen. More specifically, the invention refers a part of the non-collagenous domain of the α3 chain of type IV collagen having intact cysteine residues, said part being recognized by circulating autoantibodies from Goodpasture patients. The invention also refers to methods for the *in vitro* determination in body fluids of circulating autoantibodies from Goodpasture patients.

Goodpasture's syndrome is a prototype autoimmune disease characterized by the formation of pathogenic autoantibodies directed against the basement membrane collagen type IV. The formation of autoantibody-antigen complex in the glomerular basement membrane causes a rapidly progressive glomerulonephritis often accompanied by life-threatening pulmonary hemorrhage.

Since Goodpasture's syndrome is a classic autoimmune disease it fulfills all criteria in analogy to Koch's postulate for infectious diseases. An adaptive immune response to a self antigen causing the observed pathology is evidenced by the transfer of the disease by autoantigen specific lymphocytes or antibodies. The pathogenic role of the B cell response to the basement membrane antigen is supported by the passive transfer of the disease to monkeys with human autoantibodies eluted from the kidneys and by the relative importance of the removal of the circulating antibodies by plasma exchange in addition to the application of immunosuppressive drugs in standard treatment protocols.

The target of the toxic autoantibody response is the non-collagenous domain of the α3 chain of type IV collagen (α3(IV)NC1), but not the homologous region of the α1 chain (α1(IV)NC1). The epitope has been localized to α3(IV)NC1

which is a major basement membrane component of glomeruli and alveoli; thus explaining the tissue distribution of the disease.

In 1984 the antigen was identified as a new subunit of the NC1 domain of type IV collagen (Wieslander J. et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:3838). This resulted in the cloning of the antigen (Saus J. et al., 1988, J. Biol. Chem. 263:13374) and the designation as the α3(IV) chain. The epitope has been characterized as a cryptotope, i.e. a denaturation of the antigen increases the reactivity with human sera. However, upon reduction the reactivity disappears (Wieslander J. et al., 1985, J. Biol. Chem. 260:8564), thus indicating a three dimensional epitope that normally is hidden inside the intact glomerular basement membrane. The epitope has then been further studied and the reactivity of patient auto-antibodies has successfully been blocked with monoclonal antibodies (Pusey C. D. et al., 1987, Lab. Invest. 56:23; Hellmark T. et al., 1994, Kidney Int. 46:8263), indicating a limited epitope recognition. One study has identified the C-terminal end of the α3(IV)NC1 domain as a candidate epitope using synthetic peptides (Kalluri R. et al., 1991, J. Biol. Chem. 266:24018). Others have pointed out the importance of the amino-terminal region of the NC1 domain (Kalluri R. et al., 1996, J. Biol. Chem. 271:9062). The α3(IV) procollagen precursor sequence has been determined and is accessible from the Swiss-Prot database under AC:Q01955

A variety of studies have been performed on experimental animal models of autoimmune diseases with the aim to identify antibody epitopes of potential pathogenic relevance. It has been shown (Hellmark T. et al., 1998, Kidney Int. 55:936) that the structural requirements essential for recognition by all patient sera might be localized in the amino-terminal portion of the α3(IV)NC1

domain. However, some sera also reacted with the central portion of α3(IV)NC1.

Little is known about epitopes in human autoimmune diseases at a molecular level. This is most likely due to the methodological problems related to a diverse reactivity pattern of the autoantibodies. For example, the cysteine residues are critically involved in the folding of the NC1 domain of type IV collagen (Siebold B. et al., 1988, Eur. J. Biochem. 176:617). The reactivity of antibodies to the antigen is usually abolished if disulfide bonds are disrupted. This has for many years prevented the localization of the epitope within the NC1 domain by enzymatic digestion and such attempts have resulted in the loss of antigenicity.

Furthermore, the conformational requirements of the epitopes limit the application of linear synthetic peptides for the mapping strategies. In such studies one is limited to the use of areas of conformational flexibility such as the N-terminal and C-terminal regions because these regions of the native protein are assumed to exhibit the same spectrum of conformations as the synthetic peptide. A difficulty with this approach, however, is that due to the multiplicity of possibilities, antibody production against a desired conformation may represent a small portion of the antibodies raised and the process is relatively inefficient.

It is often of a great diagnostic benefit to determine quickly and safely the presence in body fluids of autoantibodies indicating Goodpasture's syndrome. Since the prognosis often is bad and often implies death of the patient a ready and effective diagnostic method is needed for determining whether these autoantibodies are present.

The object of the invention is to establish the target structure of the toxic antibody response in Goodpasture's syndrome, i.e. the conformational epitope

recognized by circulating autoantibodies from Goodpasture patients. On the basis of this target structure a method for a safe and accurate diagnosis, prognosis as well as therapy of Goodpasture's syndrome can be performed.

In order to achieve this purpose the method according to the invention has been given the characterizing features of claim 1.

In order to further explain the invention reference is given to the accompanying drawings in which

FIG 1 schematically illustrates the cloning strategy used to construct recombinant chimeric molecules of the amino-terminal domain of α3(IV)NC1;

FIG 2 shows immunoblotting with serum from one patient (panel A) and anti-type X collagen monoclonal antibodies (panel B) against four of the different chimeric proteins;

FIG 3 shows the immunoreactivity of a chimeric construct with sera from Goodpasture patients and healthy controls;

FIG 4 shows inhibition ELISA with recombinant chimeras blocking the reaction of patient sera to native purified α3(IV)NC1; and

FIG 5 schematically illustrates the α3(IV)NC1 domain with disulphide bonds and the 9 amino acid residues identified as the Goodpasture epitope.

In order to circumvent the above-mentioned problems an experimental procedure was chosen which allows for the expression of the antigen as a recombinant protein in a human cell line. This strategy enables the construction of a variety of properly folded chimeric molecules, in which the α3(IV)NC1 sequence harboring the Goodpasture epitope can be replaced to a varying extent by the corresponding sequence from the homologous α1(IV)NC1 that is not recognized by the toxic autoantibodies in Goodpasture's syndrome.

Since the toxic autoantibody response is highly selective for the α3-chain of the heterotrimeric type IV collagen its immunoreactive domains were subjected to a mutation analysis by the replacements with corresponding sequences from the non-reactive highly homologous α1 chain in chimeric recombinant constructs. The high homology between both chains as well as conserved disulfide bonds enhanced the likelihood for the proper folding of the hybrid constructs as prerequisite for conformation dependent autoantibody binding.

The application of this strategy for the mapping of the Goodpasture epitope revealed nine amino acid residues in the amino terminal part of the α3(IV)NC1 as the target structure recognized by all sera of 20 well defined patients with biopsy proven disease and thoroughly documented clinical outcome. Introduction of these nine amino acid residues from the α3(IV) chain of NC1 into the wild type α1(IV) sequence by replacement mutations converted the nonreactive α1(IV)NC1 into a recombinant hybrid molecule that was recognized by all patient sera with the same affinity as the native purified Goodpasture antigen as shown by competition ELISA.

The identified nine amino acid residues are localized in the amino-terminal part of the α3(IV)NC1 and form a discontinuous epitope in close vicinity to the cysteine residues that are critically involved in the folding of the NC1 domain (Siebold B. et al., 1988, Eur. J. Biochem. 176:617). The formation of disulphide bonds between the cysteine residues stabilizes the tertiary structure in the native NC1 domain and is expected to bring the identified amino acid residues more closely together. Conversely, it is very likely that the break of the disulfide bonding will affect the position of the critical residues dramatically which is in good agreement with earlier experimental

results that demonstrated the loss of immunoreactivity upon reduction of the Goodpasture antigen.

The close correlation between the specificity of the autoantibody response and clinical outcome indicates the presence of a conformational B-cell epitope which is recognized by circulating autoantibodies from apparently all Goodpasture patients. For the identification of the conformation dependent clearly pathogenic and critical epitope dominant negative mutations were introduced into the recombinant chimeric Goodpasture antigen by replacement of wild type α3(IV)NC1 sequences by corresponding amino acid residues of the homologous non-reactive α1(IV)NC1. Finally, the substitution of wild type α1(IV)NC1 in nine discontinuous positions with amino acid residues from the α3(IV)NC1 sequence resulted in a recombinant construct that was recognized by sera from all affected patients but in none of the healthy controls. The amino acid replacements define the target structure of the toxic antibody response in Goodpasture syndrome.

According to the invention the immunogenic region of the non-collagenous domain of the α3 chain of type IV collagen with intact cysteine residues is localized in the amino terminal end of the α3 chain, and at least five essential amino acid residues are exposed to circulating autoantibodies from Goodpasture patients. Four additional amino acid residues make up a conformational epitope of not more than nine amino acid residues, which is recognized by all autoantibodies from apparently Goodpasture patients. These nine amino acid residues correspond to amino acid no 1455, 1456, 1457, 1459, 1462, 1465, 1466, 1469, and 1495, respectively, in the α3(IV) procollagen precursor sequence. The amino acids in this amino terminal end of α3(IV)NC1 have been identified as L-threonine, L-alanine, L-isoleucine, L-serine, L-glutamic acid, L-valine, L-proline, L-serine, and L-glutamine, respectively.

The immunogenic region according to the invention can be obtained by chemical or enzymatical cleavage of the native non-collagenous domain of the α3 chain of type IV collagen.

Recombinant chimeric molecules of the amino-terminal domain of α3(IV)NC1 have been constructed. The proper antigen is expressed in a suitable human cell line and subsequently block the reaction of patient sera to native purified α3(IV)NC1. Recombinant molecules can then be used in a wide variety of applications.

The invention also refers to synthetic or recombinant polypeptides having as an antigenic region an amino acid sequence comprising between five and nine amino acid residues exposed to circulating autoantibodies from Goodpasture patients. Polypeptides according to the invention are sufficiently short to be synthesized by means of chemical methods now standard in the art. Such polypeptides comprise no more than about 50-60 amino acid residues, several or all of the amino acids L-threonine, L-alanine, L-isoleucine, L-serine, L-glutamic acid, L-valine, L-proline, L-serine, and L-glutamine in the polypeptide being capable of forming an epitope which immunoreacts with these autoantibodies. Further polypeptides are also contemplated as being within the scope of the present invention, which polypeptides comprise the same epitope as a polypeptide having an amino acid sequence not significantly different than that represented by the amino-terminal domain of the α3(IV) sequence shown in FIG 5. Such differences can easily be determined by, for example, competitive immunoassay.

Recombinant or synthetic molecules harboring the immunogenic region according to the invention can then be used in a number of therapy forms in relation to Good-pasture's syndrome. For example, autoantibodies directed against the basement membrane collagen type IV can be

extracorporeally eliminated by means of immunoadsorption. The epitope is immobilized by means of known techniques to a column with an inert material and a biological liquid, preferably blood plasma, is pumped through a system comprising the affinity column.

An antigen composition comprising the immunogenic region according to the invention, which specifically reacts with autoantibodies present in body fluids from Goodpasture patients, can be used for blocking the antibodies in the blood. The toxic autoantibodies can also be blocked by means of direct injecting to Goodpasture patients the epitope in the form of recombinant or synthetic molecules, an autoantibody-antigen complex being formed *in vivo* between the autoantibodies and the epitope instead of between the autoantibodies and the glomerular basement membrane. The patient is treated in this way for two weeks while waiting for diminution of the immune response by the immune complex being removed in the liver.

Autoantibody producing cells can also be killed by targeting these cells with an epitope-toxin complex. In this form of therapy a toxic substance to the cells is coupled to the recombinant or synthetic molecules according to the invention. The epitope binds to the cells and thus selectively affects them by releasing the toxic substance to the cells.

The invention also refers to methods for the *in vitro* determination in body fluids of circulating autoantibodies from Goodpasture patients. The conditions are such that they permit an antigen antibody reaction which can be demonstrated by means of standard physical or chemical means.

In such a method the inventive immunogenic region is brought into contact with a body fluid from a Goodpasture patient, which comprises circulating autoantibodies directed against the basement membrane collagen type IV.

Preferably, a solid phase system is used. The solid phase can be a plastic surface, beads for aggregation or counting, paper, nitrocellulose filters etc.

In this connection the immunogenic region is coupled to the inert carrier material in such a way that the immunogenic properties are not interfered with. The immunogenic region is preferably an integral part of the above-mentioned recombinant or synthetic molecules.

In the test systems synthetic or recombinant polypeptides as well as enzymatically or chemically cleaved products of the native protein containing the immunogenic region according to the invention are coupled to the solid phase. Alternatively, these small immunogenic molecules are captured by monoclonal or polyclonal antibodies directed against and reacting with the immunogenic region according to the invention, which in turn are coupled to the solid phase. A monoclonal antibody against the labelled immunogenic region can also be used in an inhibition assay.

Anti-idiotypic antibodies raised against the above-mentioned antibodies are likewise coupled to solid phase for capturing circulating autoantibodies from Goodpasture patients. Anti-idiotypic antibodies are thus used for detecting patient antibodies or for assaying the epitope in a sample by means of competitive assay or double sandwhich assay. In this second method anti-idiotypic antibodies raised against poly- or monoclonal antibodies directed against the inventive immunogenic region are brought into contact with a body fluid from a Goodpasture patient, which comprises circulating autoantibodies directed against the basement membrane collagen type IV.

After the addition of patient serum the system is washed. The conditions are such that they permit an antigen antibody reaction which can be demonstrated by means of standard physical or chemical means.

The detektion system can be enzymatic, flurescens, radioactivity, beads aggregated with a secondary antibody, all specific details in the test systems being well known for the skilled man within the art.

Poly- or monoclonal antibodies directed against and reacting with the immunogenic region according to the invention can readily be produced by a man skilled in the art. Likewise, anti-idiotypic antibodies raised against these antibodies should also be within the field of expertise for a man skilled in the art. Such anti-idiotypic antibodies can for example be used for preventing an immunoglobulin from combining with the basement membrane antigen.

It is also believed that it is within the qualifications of a man skilled in the art to design a diagnostic kit by means of previously known standard components and poly- or monoclonal antibodies directed against the immunogenic region according to the invention. A kit comprising recombinant or synthetic molecules with the immunogenic region according to the invention should likewise lie within his normal field of expertise.

EXAMPLES.

For the construction of recombinant chimeric molecules that harbor the Goodpasture epitope the amino terminal two thirds of the α1 chain of type X collagen including the leader sequence were fused to the NC1 domain of type IV collagen. The cloning strategy used to construct recombinant chimeric molecules is shown in FIG 1 under a. (For experimental details see below and Table 2). In FIG 1 b denotes a sequence comparison of the α3(IV)and α1(IV) chains in the amino terminal part of the NC1 domain. Capital letters indicate borders of the constructs in the regional mapping. Amino acid residues are defined by means of the single letter code. Numbers indicate the positions

(P1-P14) selected for replacement mutations. (For selection criteria see below).

The epitope mapping strategy with these constructs comprises four subsets of experiments: first the NC1 domain was divided into three parts and replaced the α3(IV) sequence by the corresponding α1(IV) sequence for each part separately; second the immunoreactive sequence was further narrowed down in the amino-terminal part of the α3(IV)NC1 domain; third point mutations were introduced in the α3(IV)NC1 in order to identify replacements with dominant negative effects on antibody recognition; and finally amino acid substitutions were performed in the α1(IV)NC1 at selected positions that reconstitute the Goodpasture epitope in a domain that is normally not recognized by the patient sera.

Therefore further mapping studies remained confined to the amino-terminal part of the α3(IV)NC1 using chimeric constructs in which the central and carboxyterminal parts of the NC1 domain were expressed as α1(IV).

Patients and sera.

Twenty samples with biopsy proven anti-GBM nephritis from the serum bank at the Department of Nephrology, Lund University were chosen and further analyzed in this study. All the patients had crescentic glomerulonephritis with linear deposits of IgG in direct immunofluorescence. Seven of the patients had in addition overt lung hemorrhage. Sera from 10 healthy blood donors were used as controls.

Antibodies.

The antibodies used were monoclonal antibodies against type X collagen (clone X53) (Girkontaite I. et al., 1996, Matrix Biol. 15:231), and the Mab17 monoclonal antibody raised against the α3(IV)NC1 (Johansson C. et al., 1991, Connect Tissue Res. 25:229).

Cell culturing and transfection.

HEK-293 cells were cultured in 90 mm cell culture plates (Nunc, Roskilde, Denmark) in a DMEM:F12 1:1 medium with 5 percent fetal calf serum (Gibco-BRL, Paisley, GB). For each construct 5 µg of the linearized plasmid DNA was transfected into 1×10^6 HEK-293 cells using an electroporator (BioRad, Hercules, CA), with the electrical settings 200V, 640µF in a 0.4 cm cuvette. The transfected cells were seeded on a new plate and after 48 hours selection was started by supplementation with 800 µg/ml G-418 (Gibco-BRL, Paisley, GB). The medium was renewed every two days; the collection of supernatants was started when the G-418 resistant cells reached confluence. During the harvesting, the transfected cells were kept in FCS-free DMEM/F12 supplemented with ascorbate (Schulte S. et al., 1998, J. Biol. Chem. 273:1551).

DNA constructs.

All restriction enzymes as well as ligase were purchased from Boehringer-Mannheim, Mannheim, Germany. The primers used are shown in Table 1 below, and the Pfu-DNA polymerase was purchased from Stratagene.

Table 1. Primers used.

Primer no	Sequence 5' to 3'
1	CATGCTGGATCCGGGGCCCCCAGGCACCA
2	GCTGGTGTTGACAGCCAGTACGAGTACTCATT
3	CTGGCTGTCAACCCCTGAGCCCATGCCCATGTC
4	TGACATTCTAGAAATGGCGCACTTCTAAACTCC
5	CCATTTCTAGAGTGTCACGGCCGTGGGACCTGC
6	CCATTGCGGCCGCTTATGTTCTTCTCATACAGAC
7	CCAGGGCCTCCTGGCTTTGGG
8	GGCATTCCAGGATTCCCTGGG
9	TCATGTCCAGAGGGGACAGTC
10	TGTCCCCTCTGGACATGATGGGTCATCTATTGTTTGAC
11	CCACAGTGTCCTTCTGGGACC
12	CCCAGAAGGACACTGTGGAATTGCTGTGGTTTGACTGTG
13	ACAGTGCCACTCTACAGTGGGTACTCTTTGCTCTACG
14	ACTGTAGAGTGGCACTGTCC
15	GGCAGCTGCCTGCAGCGATTCAGCACAATGCCCTTCC
16	TCGCTGCAGGCAGCTGCC
17	CCAGGCACCCCATCTGTTACGAGAGGCTTTGTCTTC

(Table 1. cont.)

18	AACAGATGGGGTGCCTGG
19	ACCAGGCATAGTCAAACAACAGCAATTCCTTCATGTCC
20	TGTTTGACTATGCCTGGTC
21	GATCCACTGCTTCCACGTTT
22	GTTGTCCAGGTTCCAGGTGATCC
23	GAAGACAAAGCCGTGCGTTGTCCA
24	GTGTCGGGTGACGACAAAGCC
25	GAAGGAATATCTGTGGTTTGACp5
26	GGACATGAAGGATCTGCTGTGG
27	CCCTCTGGACATTGAGGAATTGC
28	GGCACTGTCCCCGATGGACATG
29	CCACTGTAGAGTATCACTGTCCC
30	GAAAACCCATGGTAGAGTGGC
31	TTCCTTGTACATAAAGAAAAGA
32	AGGCAGCTGCCAGCAGTTCCAAG
33	GGTAAATCGCGCAGGCAGCT
34	GGCATTGTGCTAAATCGCTGC
35	ACAATAGATATCCCATCGTGTCCTTCTGGG
36	CCCAGAAGGACAGGATGGGATATCTATTGTTTG
37	CTGGGACCAAACCTCTTTACAGCGGGTACTCTTTGC
38	GCAAAGAGTACCCGCTGTAAAGAGGTTTGGTCCC
39	AGCTGCCTGCAAAAGTTCAGC
40	GCTGAACTTTTGCAGGCAGCTGC

Construction of primary vectors.

The primary type X/type IV collagen chimera was constructed using the α3(IV)NC1 cDNA cloned in pBluescript SK-vector (Stratagene, LaJolla, CA) with a BamHI site in the 5' end and a NotI site in the 3' end, and the full length type X collagen cDNA in the pBluescript SK-vector cloned between the HindIII and NotI sites. Both plasmids were cleaved with BamHI and NotI and the α3(IV)NC1 cDNA was ligated into the internal BamHI site of type X collagen cDNA. This construct contains five unique restriction enzyme sites; a HindIII site in the 5' end of the construct, a BamHI site in the junction between the type X collagen and the type IV collagen NC1, HindII and XbaI in the α3(IV)NC1, and a NotI site 3' of the coding DNA.

Dividing of the NC1 domain in three parts.

The BamHI, HindII, XbaI and NotI cleavage sites have been introduced into the α1(IV)NC1 cDNA by PCR)Perkin-Elmer GeneAmp 2400, Foster City, CA), using primers 1-6.

Upon restriction with the appropriate enzymes the fragments of the α1(IV)NC1 were used to replace the corresponding cassettes of the wild type α3(IV) sequence as previously described (Hellmark et al., 1998, Kidney Int. 55:936). Since the major epitope region could be localized to the amino-terminal domain of the α3(IV)NC1 this region was subjected to further mutational analysis. For the construction of six α1/α3(IV) chimeric NC1 domains the overlap extension PCR technique (Ho S. N. et al., 1989, Gene 77:51) was applied using the primers 6-20 in Table 1.

Replacement mutations of single amino acid residues in the α3(IV) sequence.

Fourteen positions were selected for mutational analysis in a chimeric construct that harbors the amino-terminal α3(IV)NC1 (from A to G in Figure 1). The selection criteria were based on identity in human and bovine α3(IV) sequences and concurrent non-conserved exchanges in the corresponding amino acid residues of the human α1(IV) and α5(IV) sequences. These 14 amino acid residues are emphasized in FIG 1 by the numbering of their positions in the sequence (1-14). The point mutations were introduced by the megaprimer method (Barik., S., 1993, In Methods in Molecular Biology, Vol. 15, B. A. White editor, Humana Press Inc., Totowa, New Jersey, 277-286) using the primers 6, 7, and 21-34.

Replacement mutations of single amino acid residues in the α1(IV) sequence.

Finally, replacement mutations were introduced into chimeric constructs comprising the entire α1(IV)NC1 domain. By site directed mutagenesis 5 and 9 codons were changed from wild type α1(IV) to the corresponding α3(IV), respectively. The substitutions were introduced by an overlap extension PCR (Ho S. N. et al., 1989, Gene 77:51)

using primers 6, 7 with 35-40 and primers 6, 7 with 15, 16, 19, 20, 39 and 40 respectively.

General handling of the constructs.

After sequencing, the constructs were restricted with HindIII and NotI and subcloned into a CMV promoter driven expression vector (pcDNA3, Leek, The Netherlands). All constructs were tested for translation of a protein with correct molecular weight using an *in vitro* system (Promega, Madison, WI), with a S^{35} labeled cysteine and T7RNA polymerase. Before transfection, the plasmid DNA was linearized using ScaI.

Enzyme Linked Immunosorbent Assay (ELISA).

Native type IV collagen NC1 domains were purified as described elsewhere (Hellmark T. et al., 1994, Kidney Int. 46:8263), and coated at 0.5 µg/ml. The coating efficiency of the different recombinant proteins was calibrated by their equal immunoreactivity with the anti-collagen type X monoclonal antibody. Human sera were diluted 1/100 and the monoclonal antibodies 1/1000. The ELISA was performed following standard procedures (Hellmark T. et al., 1994, Kidney Int. 46:8263) using alkaline-phosphatase conjugated swine anti-human IgG (Orion Diagnostica AB, Trosa, Sweden) as secondary antibody. For a positive ELISA, the absorbency value had to be higher than the mean value for the control sera plus two standard deviations. None of the control sera were positive in any ELISA.

Inhibition ELISA.

Dilutions of human sera were adjusted to give the same absorbancy after one hour in a conventional ELISA with purified native type IV collagen NC1 domains. They were preincubated overnight at 4°C with different inhibitors; i.e. recombinant proteins or purified native proteins in concentrations from 0.0025 to 25 µg/ml. The amount of IgG that reacts with the surface bound Goodpasture antigen

despite the presence of inhibitor molecules in the fluid phase was determined after extensive washing procedures with alkaline-phosphatase-conjugated secondary antibodies as described above.

SDS-PAGE and immunoblotting.

One ml of the cell supernatants was precipitated with Triton X100 and trichloro acetic acid. The precipitate was applied to SDS-PAGE in 10-16 % gradient gels (SDS-PAGE) under nonreducing conditions (Laemmli U. K., 1970, Nature 227:680).

Immunoblotting experiments were performed on samples that were separated with SDS-PAGE and transferred to Immobilon™PVDF membrane (Millipore, Saint-Quentin, France) (Burnette W. N., 1981, Anal. Biochem. 112:195).

Example 1. Replacement of subdomains of the amino-terminal portion of the α3(IV)NC1 by the corresponding α1(IV) sequences.

In order to further narrow down the sequence that harbors the major epitope, six different constructs were generated that contain replacements of different parts of the amino-terminal domain of α3(IV)NC1 by the corresponding sequences from the α1(IV). These six chimeric proteins were designated D2 to D7 as shown in Table 2 below.

Table 2. Construction design (see FIG 1 for nomenclature and amino acid positions).

Name	Construct	Number of immunoreactive sera *
Regional Mapping (Division of the NC1 domain)		
D1	α3 from A to G then α1	20/20
D2	α1 from A to B then α3 to G then α1	20/20
D3	α1 from A to C then α3 to G then α1	20/20

(Table 2. cont.)

D4	α1 from A to D then α3 to G then α1	0/20
D5	α3 from A to E then α1	20/20
D6	α3 from A to F then α1	4/20
D7	α3 from A to D then α1	0/20
Replacement Mutations aimed at destroying the epitope (Substitutions in the α3(IV)NC1)		
P1	α3 from A to G then α1; Substitution in position 1, D to S	20/20
P2	α3 from A to G then α2; Substitution in position 1, A to G	20/20
P3	α3 from A to G then α3; Substitution in position 1, R to H	20/20
P4	α3 from A to G then α4; Substitution in position 1, F to V	20/20
P5	α3 from A to G then α5; Substitution in position 1, A to D	20/20
P6	α3 from A to G then α6; Substitution in position 1, I to D	7/20
P7	α3 from A to G then α7; Substitution in position 1, S to Q	5/20
P8	α3 from A to G then α8; Substitution in position 1, E to S	20/20
P9	α3 from A to G then α9; Substitution in position 1, P to I	0/20
P10	α3 from A to G then α10; Substitution in position 1, S to H	0/20
P11	α3 from A to G then α11; Substitution in position 1, F to Y	20/20
P12	α3 from A to G then α12; Substitution in position 1, L to A	20/20

(Table 2. cont.)

P13	α3 from A to G then α13; Substitution in position 1, Q to R	4/20
P14	α3 from A to G then α14; Substitution in position 1, T to S	20/20
Reconstruction of the epitope substitutions in the α1(IV)NC1)		
S1	α1 from A; Substitutions in position 6(D to I),7(Q to S),9(I to P),10(H to S),13(R to Q)	0/20
S2	α1 from A; Substitution in position 5(D to A),6(D to I),7(Q to S),8(S to E),9(I to P),10(H to S),13(R to Q) and in addition I to T a.a.35,K to V a.a.45	20/20

* n = 20 patients with biopsy-proven Goodpasture disease. The immunoreactivity was assessed by ELISA experiments (see above for details).

It was found that all sera (n=20) reacted with the D2, D3, and D5 chimeric proteins while no antibody binding was detectable to D4 and D7. A subset of 4 sera also reacted with the D6 chimeric protein. The reactivity of the Goodpasture sera with the constructs indicates the localization of the major epitope to a region comprising the 42 amino acid residues between the positions C and F in FIG 1. However, for 20 percent of the sera the requirements for antibody recognition remained confined to the 15 amino acid residues of α3(IV) from C to E in FIG 1.

Example 2. Amino acid substitutions in the α3(IV)NC1 with dominant negative effect on autoantibody recognition.

Comparison of amino acid sequences for the α1(VI) and α3(IV) chain revealed 33 non-conserved residues in the

amino-terminal portion of the NC1 domain from position A to G in FIG 1. For the identification of those amino acid differences that might be of critical importance for antibody binding the sequence comparison was extended to bovine α3(IV) and human α5(IV) sequences as bovine α3(IV) is recognized by autoantibodies from Goodpasture patients in contrast to the non-reactive human α5(IV). Only positions with identity in the human and bovine α3(IV) sequences but with differences to the corresponding α1(IV) and α5(IV) sequences were selected for the introduction of replacement mutations. This criterion was fulfilled by 14 amino acid residues, and their positions are indicated by the numbering of the positions (1-14) in FIG 1. In these positions the α3(IV)-specific codons were replaced by the corresponding α1(IV) residues in the construct D1 (Table 2), resulting in different chimeric molecules that harbor a single amino acid exchange, named P1 - P14 in Table 2. Small differences in reactivity with the recombinant proteins were obtained with sera from different individuals. However, two of the constructs, i.e. P9 and P13, at lest partially abolished their reactivity. The results concerning the localization of critical positions for immunoreactivity by single amino acid residue substitutions are in concordance with the effect of replacement mutations of longer stretches of the α3(IV) sequence by the corresponding α1(IV) domains in the aminoterminal portion of NC1. Thus, all positions that turned out to be of essential importance localize within the same 42 amino acid residue long region (between C and F in FIG 1) that has been shown in the above described experiments to require α3(IV) sequences for binding of the Goodpasture sera. In contrast to the majority of Goodpasture patient samples (n=16) the reactivity of the four sera, which remained insensitive to the Q to R mutation in the P13 construct, had earlier been shown to be

restricted to a subdomain of the critical region (from C to E in FIG 1) which does not include position P13.

Example 3. Construction of the Goodpasture epitope by site-directed mutagenesis in the α1(IV)NC1.

Based on the knowledge of positions in the α3(IV)NC1, in which single replacement mutation can destroy the immunoreactivity, the Goodpasture epitope was reconstructed within the frame of the non-reactive α1(IV)NC1 by site-directed mutagenesis of five and nine amino acid residues respectively. First, the five critical residues in position 6, 7, 9, 10, and 13 with the dominant negative effect on autoantibody binding were replaced in the α1(IV)NC1 by the corresponding amino acid residues, I, S, P, S, and Q from the α3(IV) sequence (construct S1 in Table 2). These five amino acid residues are essential for the immunoreactivity of the Goodpasture epitope as will be shown below.

Second, four additional non-conserved residues localized in the region between C and E in FIG 1 were substituted. The resulting α1(IV)NC1 construct harbors all eight amino acid substitutions from the α3(IV) sequence between C and E in FIG 1 and the amino acid exchange R to Q in position P13 (construct S2 in Table 2). Likewise, as will be shown below, these four amino acid residues are important for the most efficient target structure of the antibody response in Goodpasture's syndrome but they are not essential for the immunoreactivity of the Goodpasture epitope.

FIG 2 shows immunoblotting of four different chimeric proteins with serum of a representative Goodpasture patient (panel A) and with a control monoclonal antibody (mAb; murine anti-collagen X) (panel B). Lane 1: recombinant α3(IV)NC1; lane 2: D1 construct; lane 3: S1 construct; lane 4: S2 construct. The upper row (arrowhead) indicates bands in which specific reactivity has occurred, thus indicating that the control antibody recognized all four

constructs. In contrast, the patient antibodies bound to α3(IV)NC1, D1 and S2, but not to the S1 construct containing the five amino acid substitutions which were shown to be insufficient to reconstitute ELISA-reactivity in the frame of α3(IV)NC1 (cf. Table 2 above). Bands of low molecular weight in panel A are likely due to non-specific reactivity to minor contaminants from the cell culture medium, whereas in panel B (lane 1) the bands may indicate partial degradation of the α3(IV)NC1.

The first construct, S1, gave only a weak reactivity with Goodpasture sera in immunoblotting experiments and the mutational changes were insufficient to make the molecule reactive with patient sera in ELISA. The second chimeric protein, S2, was on the other hand recognized by all sera in both immunoblotting (FIG 2) and ELISA; thereby indicating that all reactivity of the Goodpasture autoantibodies is directed towards this limited region in the NC1 domain of collagen type IV.

FIG 3 shows the immunoreactivity against the chimeric construct S2 (see Table 2 above for details) of Goodpasture sera (n=20), 13 patients without overt lung hemorrhage (filled circles) and 7 patients with lung hemorrhage (open circles), as well as age-matched controls (n=10) (open squares). ELISA experiments showed a clear distinction between the Goodpasture patients and the healthy control populations. The cut-off value (mean value of the healthy controls + 2 SD) is indicated by a horizontal line.

As shown in FIG 3 there was no difference between epitope specificities of autoantibodies from patients with or without lung bleeding when the chimeric construct S2 was used. In contrast to the 20 Goodpasture patients, there was no reactivity in the 10 sera from healthy humans used as control.

Example 4. Inhibition ELISA.

For the evaluation of the affinity of Goodpasture autoantibodies for the different chimeric proteins inhibition ELISAs have been performed using recombinant α3(IV)NC1, D1, S1, and S2 to block the binding to native NC1 domains from type IV collagen. The data confirm that the major epitope in Goodpasture's syndrome is preserved in the S2 chimeric protein.

The affinity of Goodpasture antibodies to the chimeric constructs is shown in FIG 4. Here all the inhibition ELISA curves were derived from one representative patient. The antibody binding to the native α3(IV)NC1 coupled to a solid phase was inhibited by the soluble recombinant α3(IV)NC1, D1, and S2 chimeric proteins, but not by the S1 construct. For α3(IV)NC1, D1, and S2 the decline in immunoreactivity indicates a comparable affinity below saturation levels. At saturation the inhibition capacity of D1 and S2 remained at 85 % level of that of α3(IV)NC1, thus indicating that only 15 % of the autoantibodies recognize structures not contained in the immunodominant 9 amino acid residues but which are fully contained in the S2 construct (cf. Table 2).

As seen in FIG 4, the reaction could be inhibited to 85 % with both the D1 and S2 chimeric proteins, and that the affinity is approximately the same. The recombinant α3(IV) could inhibit the reaction to 100 %, hence 15 % of the autoantibodies are directed against other areas of the α3(IV) chain than the N-terminal of the α3(IV)NC1 in this specific serum. This tendency was shown to be true for all sera, i.e. the D1 and S2 inhibited the antibody binding less than the recombinant α3(IV)NC1, nonetheless all sera had the majority of their autoantibodies directed against the epitope expressed on the S2 construct.

FIG 5 is a schematic drawing of the secondary structure of the α3(IV)NC1 domain involved in Goodpasture

autoimmunity. The immunogenic region of this domain is based on the results obtained with the chimeric construct S2 and includes two crossing disulfide bonds (indicated by solid lines between cysteine residues with roman numerals). Each amino acid of the linear sequence is represented by circles. The filled circles emphasize the critical nine amino acid residues identified, and they are all clustered in the close vicinity of the disulphide bonds of the cysteine residues I-IV. It should be noted that these bonds can exist between the cysteine residues I-II and II-IV as well as between the cysteine residues I-IV and II-III.

As shown in FIG 5 the identified critical nine amino acid residues are all localized in the amino terminal part of α3(IV)NC1 and form a discontinuous epitope in close vicinity of the those cysteine residues which are involved in the folding of the NC1 domain. The formation of disulphide bonds between cysteine residues stabilizes the tertiary structure of the native NC1 domain, the critical amino acid residues being brought into a spatial relationship which is important for antibody recognition. Conversely, it is very likely that breakage of these disulphide bonds would dramatically affect the position of the critical residues, a result which would be consistent with earlier results documenting loss of immunoreactivity upon reduction of the Goodpasture antigen.

CLAIMS

1. Immunogenic region comprising a part of the non-collagenous domain of the α3 chain of type IV collagen having intact cysteine residues, said part being recognized by circulating autoantibodies from Goodpasture patients, c h a r a c t e r i z e d in that said part comprises at least five amino acid residues which are substantially non-adjacent in the amino acid sequence, is located in the amino terminal end of said α3 chain, and is exposed to said autoantibodies.

2. Immunogenic region as claimed in claim 1, c h a r a c t e r i z e d in that said part comprises not more than nine amino acid residues.

3. Immunogenic region as claimed in claim 2, c h a r a c t e r i z e d in that said not more than nine amino acid residues corresponds to amino acid no 1455, 1456, 1457, 1459, 1462, 1465, 1466, 1469, and 1495, respectively, in the α3(IV) procollagen precursor sequence.

4. Immunogenic region as claimed in claim 3, c h a r a c t e r i z e d in that said amino acids comprise L-threonine, L-alanine, L-isoleucine, L-serine, L-glutamic acid, L-valine, L-proline, L-serine, and L-glutamine, respectively.

5. Polypeptide molecules comprising an immunogenic region as claimed in any of claims 1 to 4.

6. Polypeptide molecules comprising an immunogenic region which is not significantly different than that claimed in any of claims 1 to 4 as determined by competitive immunoassay.

7. Recombinant molecules comprising the immunogenic region as in any of claim 1 to 6.

8. Synthetic molecules comprising the immunogenic region as in any of claim 1 to 6.

9. A diagnostic kit containing immunogenic molecules as in any of claim 5 to 8.

10. Polyclonal antibodies excluding autoantibodies from Goodpasture patients, which are directed against and reacting with the immunogenic region as in any of claim 1 to 4.

11. Monoclonal antibodies directed against and reacting with the immunogenic region as in any of claim 1 to 4.

12. Polyclonal antibodies directed against and reacting with the immunogenic molecules as in any of claim 5 to 8.

13. Monoclonal antibodies directed against and reacting with the immunogenic molecules as in any of claim 5 to 8.

14. A diagnostic kit containing poly- or monoclonal antibodies as in claim 10 to 13.

15. Anti-idiotypic antibodies raised against antibodies of claim 10 to 13.

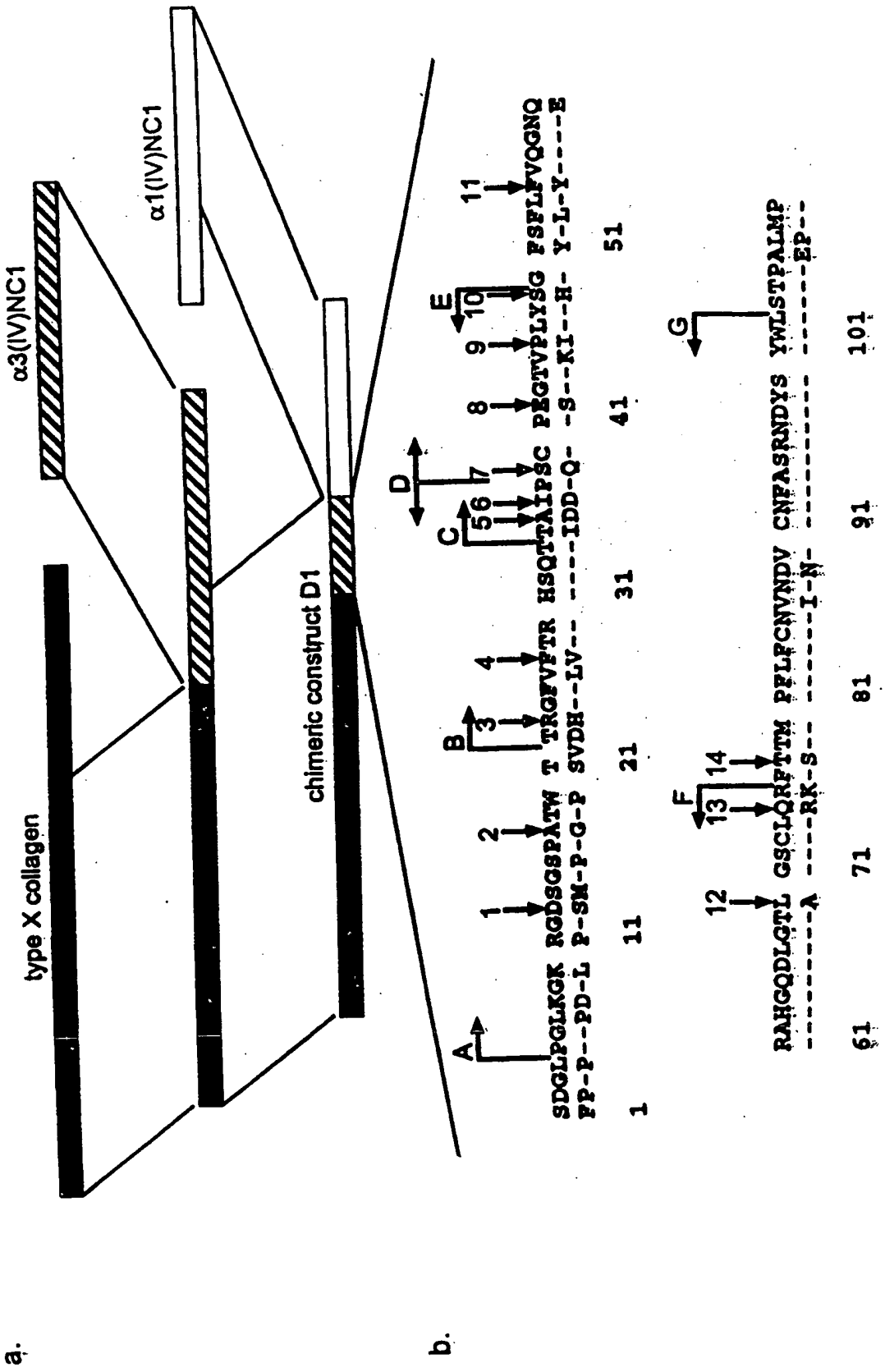
16. Antigen composition comprising the immunogenic region as claimed in any of claims 1 to 8, which specifically reacts with autoantibodies present in body fluids from Goodpasture patients.

17. Method for the *in vitro* determination in a body fluid of circulating autoantibodies from Goodpasture patients, c h a r a c t e r i z e d in that the immunogenic molecules as claimed in any of claims 5 to 8 are brought into contact with said body fluid under conditions which permit an antigen antibody reaction, said reaction then being demonstrated by means of physical or chemical means.

18. Method as claimed in claim 17, c h a r a c t e r i z e d in that said immunogenic molecules are coupled to a carrier material.

19. Method for the determination in a body fluid of circulating autoantibodies from Goodpasture patients, c h a r a c t e r i z e d in that anti-idiotypic antibodies as claimed in claim 15 are brought into contact with said body fluid under conditions which permit an antigen antibody reaction, said reaction then being demonstrated by means of physical or chemical means.

Fig. 1


a.
α3(IV)NC1
α1(IV)NC1
type X collagen
chimeric construct D1
b.
A
B
C
D
E
F
G
SDGLPGLKGK RGDSGSPATW T TRGFVPTR HSQTTAIPSC PEGTVPLYSG FSFLFVQGNQ
FP-P--PD-L P-SM-P-G-P SVDH--LV-- ----IDD-Q- -S--KI--H- Y-L-Y---E
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---------A ----RK-S-- ------I-N- ---------- -----EP--

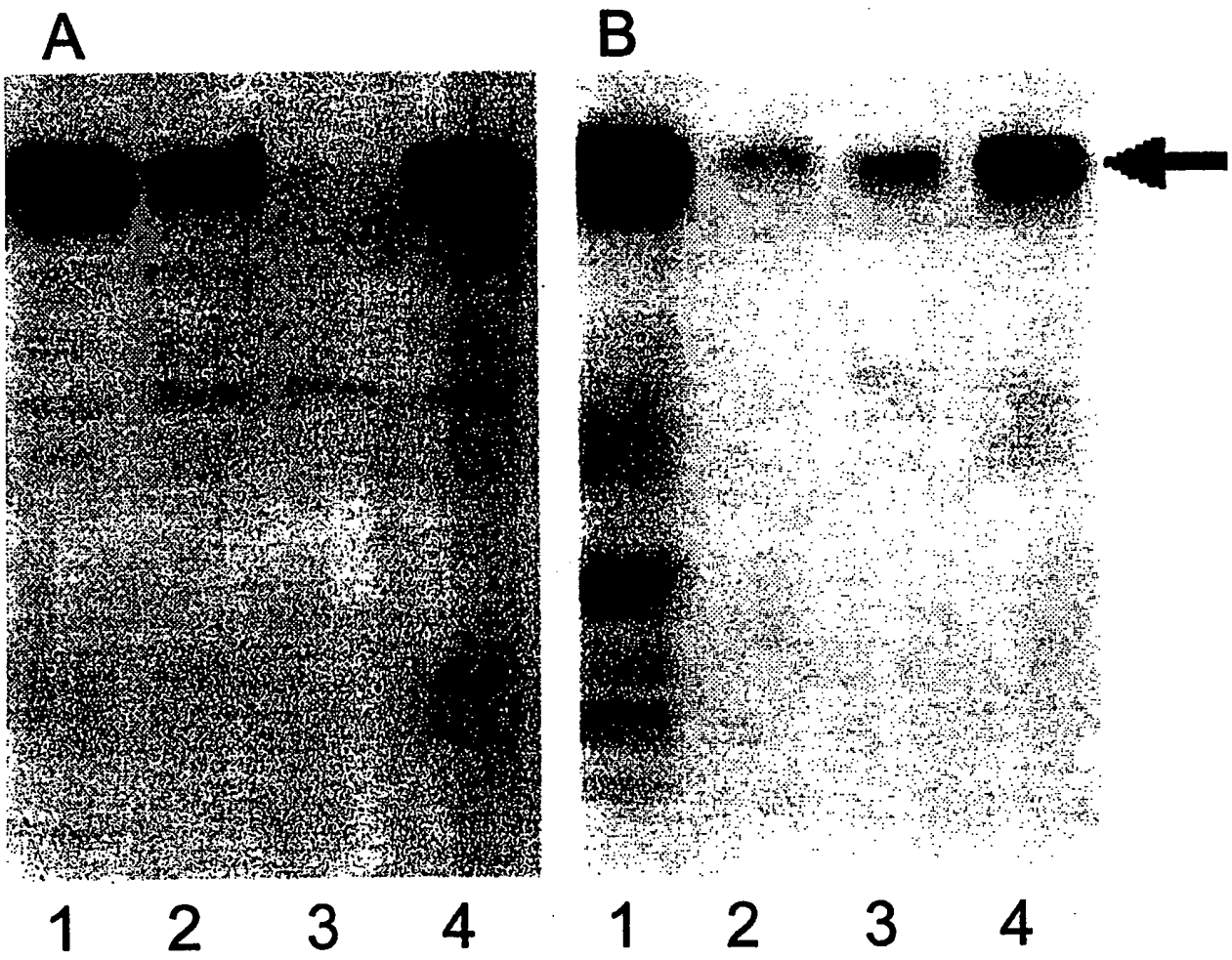

A
B
1 2 3 4
1 2 3 4

Fig. 3

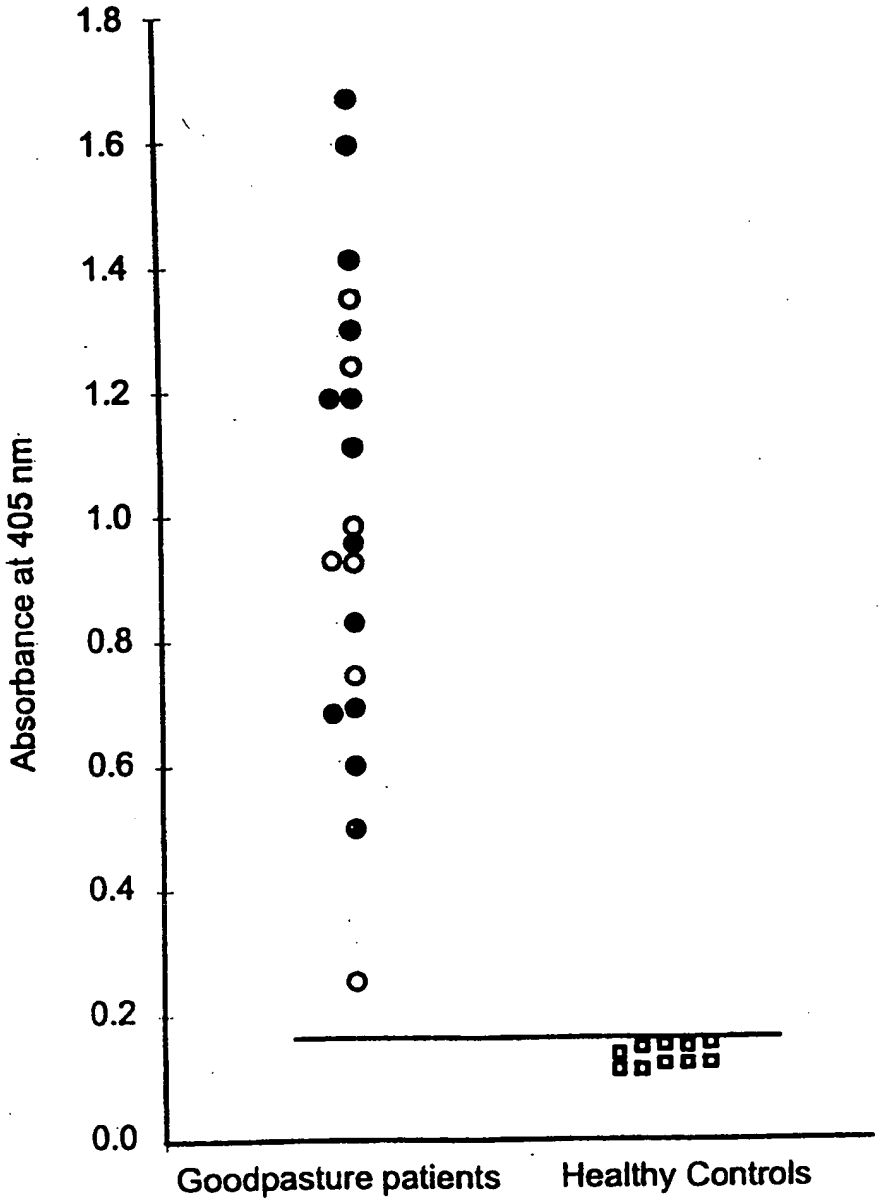

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance at 405 nm
Goodpasture patients
Healthy Controls

Fig. 4

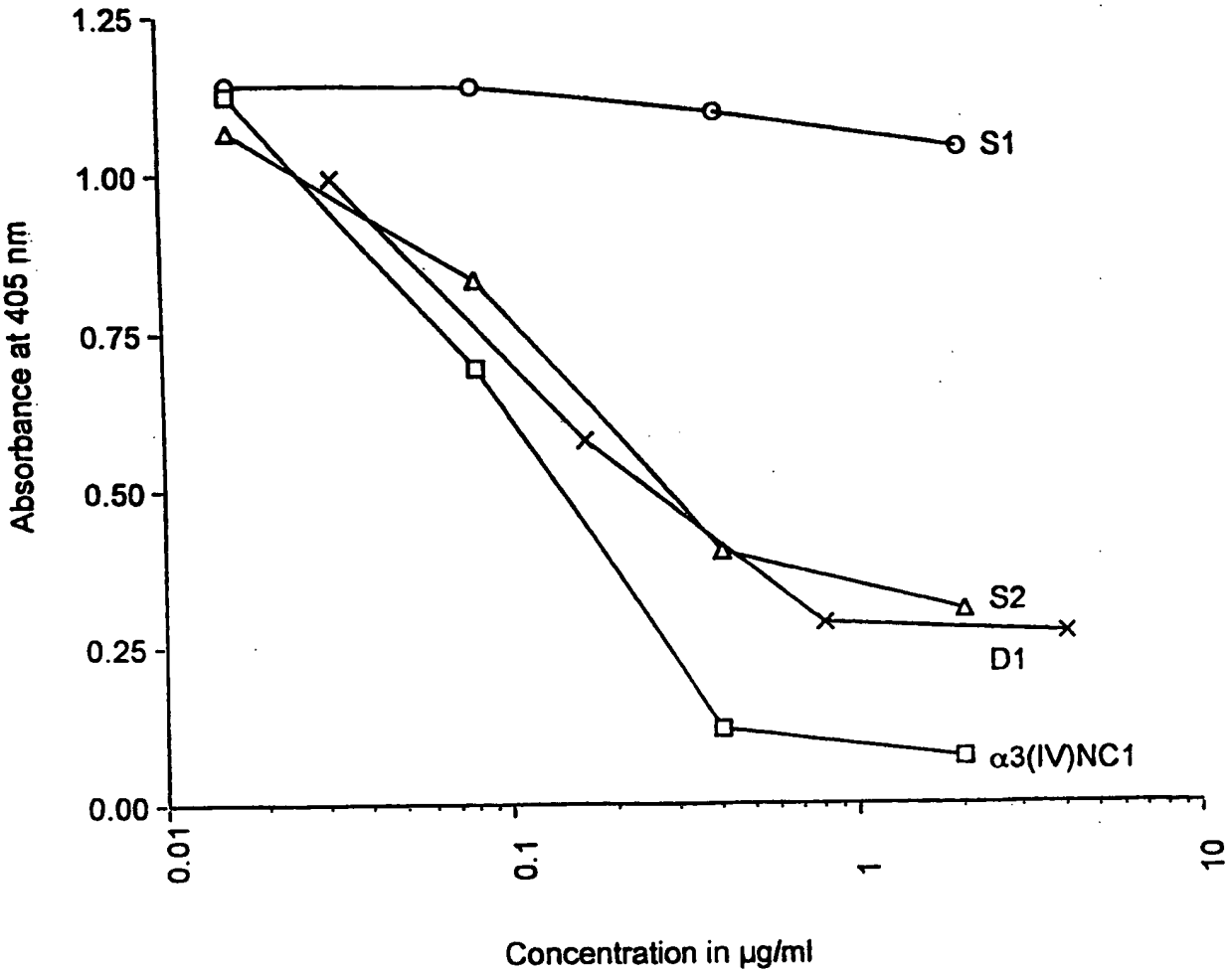
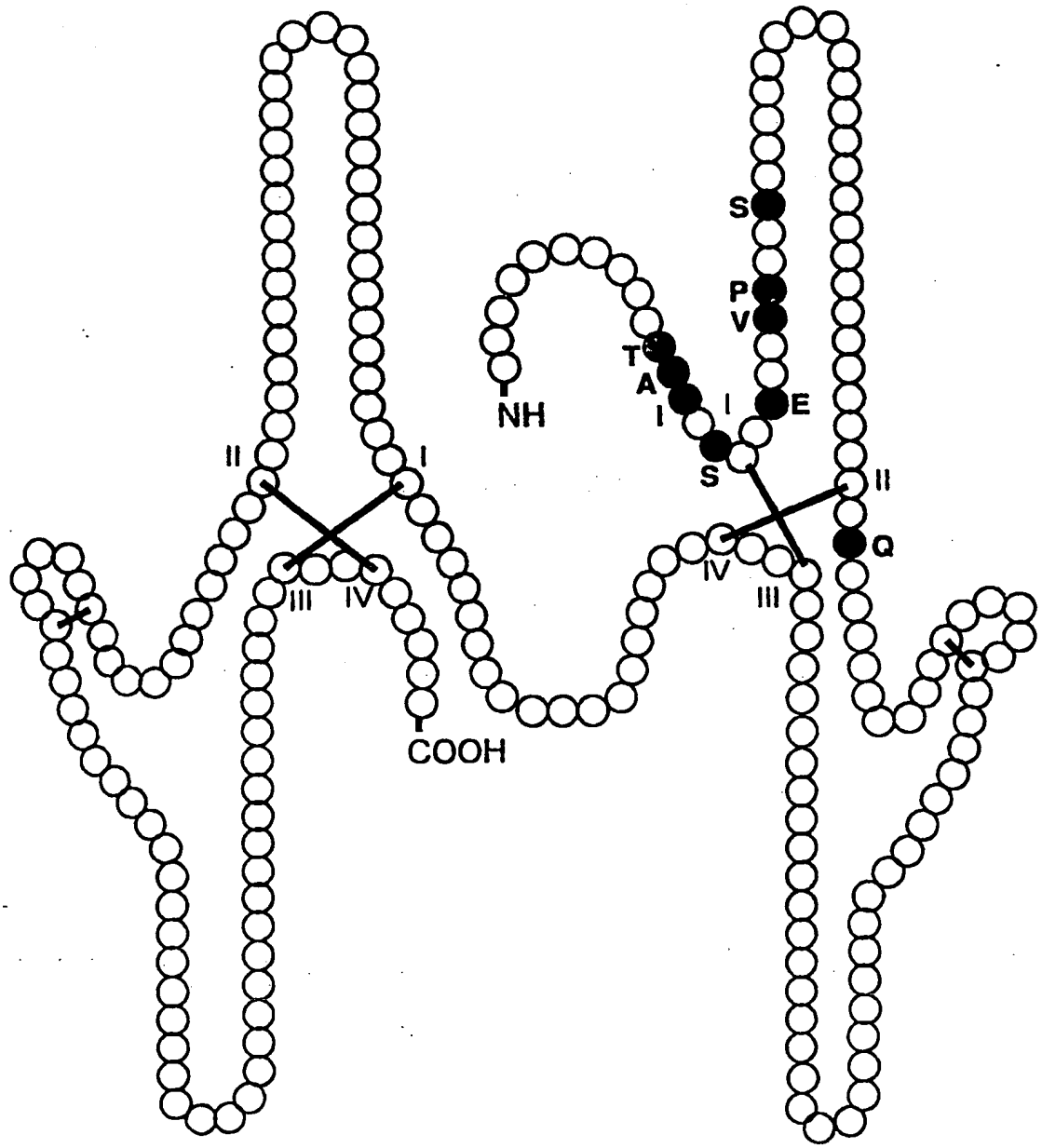

1.25
1.00
0.75
0.50
0.25
0.00
Absorbance at 405 nm
0.01
0.1
1
10
Concentration in µg/ml
S1
S2
D1
α3(IV)NC1

Fig. 5



INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/01416

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: G01N 33/564, C07K 16/18, C07K 16/18, C07K 14/78
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: G01N, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	The Journal of Biological Chemistry, Volume 271, No 15, 1996, Raghu Kalluri et al, "The Goodpasture Autoantigen. Structural delineation of two immunologically privileged epitopes on alpha3(IV) chain of type IV collagen", page 9062 - page 9068, see the"discussion"	1,2,5-14, 16-19
	--	
X	Clin Exp Immunol, Volume 113, No 17, 1998, J. J. Ryan et al, "Recombinant alpha-chains of type IV collagen demonstrate that the amino terminal of the Goodpasture autoantigen is crucial for antibody recognition", page 17 - page 27, see fig. 8 and"Discussion"	1,2,5-14, 16-19
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 December 1999	12 -01- 2000
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl-Olof Gustafsson/EÖ Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/01416

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Kidney International, Volume 53, 1998, Kevin E.C. Meyers et al, "Human Goodpasture anti-alpha3(IV)NC1 autoantibodies share structural determinants. Rapid Communication" page 402 - page 407	1,10-13,14
A	Journal of the American Society of Nephrology, 1997, ThomasHellmark et al: "Comparison of Anti-GBM Antibodies in Sera With or Without ANCA", page 376 - pages 385	1
A	US 5424408 A (STEPHEN T. REEDERS ET AL), 13 June 1995 (13.06.95)	1-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/11/99

International application No.
PCT/SE 99/01416

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5424408 A	13/06/95	NONE	